

# *kin-19*/casein kinase I $\alpha$ has dual functions in regulating asymmetric division and terminal differentiation in *C. elegans* epidermal stem cells

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Casein Kinase I (CKI) is a conserved component of the Wnt signaling pathway that regulates cell fate determination in metazoans. We show that post-embryonic asymmetric division and fate specification of *C. elegans* epidermal stem cells are controlled by a non-canonical Wnt/ $\beta$ -catenin signaling pathway, involving the  $\beta$ -catenins WRM-1 and SYS-1, and that *C. elegans kin-19/CKI $\alpha$*  functions in this pathway. Furthermore, we find that *kin-19* is the only member of the Wnt asymmetry pathway that functions with, or in parallel to, the heterochronic temporal patterning pathway to control withdrawal from self-renewal and subsequent terminal differentiation of epidermal stem cells. We show that, except in the case of *kin-19*, the Wnt asymmetry pathway and the heterochronic pathway function separately and in parallel to control different aspects of epidermal stem cell fate specification. However, given the function of *kin-19/CKI $\alpha$*  in both pathways, and that CKI $\alpha$ , Wnt signaling pathway and heterochronic pathway genes are widely conserved in animals, our findings suggest that CKI $\alpha$  may function as a regulatory hub through which asymmetric division and terminal differentiation are coordinated in adult stem cells of vertebrates.

## Introduction

Asymmetric cell division (AD) and self-renewal (SR) are defining characteristics of stem cells. In asymmetric division, the progenitor stem cell undergoes mitosis to give rise to progeny cells that have different fates. One daughter cell differentiates, thus becoming more restricted in its cell fate potential, and typically exits the cell cycle. The fate of the other daughter cell is self-renewal, that is it retains proliferative capability and maintains the cell fate potential of the progenitor cell. The cell behaviors of AD and SR allow stem cells to populate or re-populate, the differentiated cells of a tissue while maintaining multipotent stem cell lineages, and these behaviors are fundamental to the processes of both tissue formation during development and tissue regeneration and repair.<sup>1,2</sup> The *C. elegans* seam cells are multipotent epidermal stem cells that undergo AD and SR during post-embryonic larval stages, and serve as a model for investigating stem cell development.<sup>3,4</sup>

The *C. elegans* seam cells (H0-H2, V1-6, T) undergo asymmetric divisions at each of the four larval stages (L1-L4) to produce self-renewing seam cells and differentiated hypodermal cells. For most seam cell lineages, the posterior daughter cell

becomes the self-renewing seam cell while the anterior daughter cells terminally differentiate by fusing with the surrounding epidermal syncytium (Hyp7).<sup>5</sup> In the V5 and T lineages, the non-self renewing daughter cells undergo further divisions in the L2 stage and differentiate into neural cells and neuronal support cells.<sup>5</sup> The self-renewing seam cells themselves terminally differentiate at the end of larval development, as is the case for all somatic lineages in *C. elegans*. In addition to asymmetric divisions at every larval stage, the V1 to V6 seam cell lineages also undergo one round of symmetric division during the L2 stage, thus increasing the seam cell number.

Two genetic pathways have been shown to regulate seam cell development: The heterochronic pathway controls stage specific patterns of seam cell division at each larval stage, as well as seam cell loss of self-renewal at the end of larval development that results in terminal differentiation, while *C. elegans* homologs of the Wnt/ $\beta$ -catenin signaling have been implicated in the control of asymmetric division and cell fate determination of the V5.p and T seam cells.<sup>6-8</sup> Additionally, *ceb-16*, the homolog of *engrailed*, is a transcriptional regulator that represses genes involved in seam cell terminal differentiation and activates genes that maintain seam cell fate.<sup>9</sup> The L2-specific divisions are regulated by RNT-1

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and BRO-1 transcription factors, the *C. elegans* homologs of Runx and its binding partner CBF $\beta$ , respectively, which control hematopoietic stem cell development in vertebrates, and by *sel-6* (also known as *emb-4* and *mal-2*), which encodes a splicing protein that regulates germline chromosome condensation during embryonic development.<sup>10-12</sup> The heterochronic pathway and the Wnt signaling pathways function at every larval stage, and mutations in either of these pathways affect both seam cell division and differentiation patterns, raising the possibility that there is cross-regulation between these pathways.<sup>13,14</sup> In this study we sought to identify new components of seam cell Wnt signaling and heterochronic pathways, focusing especially on candidates with potential dual function in both pathways. Additionally, we sought to define the regulatory relationship, if any, between the Wnt signaling and heterochronic pathways in regulation of seam cell asymmetric division and self-renewal.

The Casein kinase I (CKI) family of serine/threonine kinases functions in  $\beta$ -catenin dependent Wnt signaling pathways, which are broadly conserved in animal species and regulate numerous aspects of cell fate determination, including stem cell development.<sup>2,15</sup> In the canonical Wnt pathway of *Drosophila* and vertebrate species, CKI $\alpha$  functions as part of a cytoplasmic 'destruction complex' that includes the scaffold proteins AXIN and APC, and GSK3 $\beta$  kinase.<sup>16</sup> This 'destruction complex' regulates the stability of  $\beta$ -catenin, which is phosphorylated in the absence of Wnt signaling and thus targeted for ubiquitylation and degradation. When Wnt signals are detected by Frizzled/LRP co-receptor complexes, and the signal is transduced through cytoplasmic Dishevelled (Dsh),  $\beta$ -catenin phosphorylation by the 'destruction complex' is inhibited allowing stabilized  $\beta$ -catenin to enter the nucleus, where it functions with Tcf/Lef transcription factors to induce expression of Wnt signaling pathway target genes.<sup>15</sup> Thus, in the current model of canonical Wnt signaling, different cell fates in the daughter cells of asymmetric division depends on differential ability to receive and transduce Wnt signals, and on the differential regulation of Wnt pathway components, such as CKI $\alpha$  and CKI $\epsilon/\delta$ , which control the stability and function of  $\beta$ -catenin and Tcf/Lef.

In *C. elegans*, the asymmetric cell fate choice that occurs upon seam cell division is also controlled by  $\beta$ -catenin dependent Wnt signaling pathways, and specifically by differential levels of POP-1, the *C. elegans* Tcf/Lef homolog.<sup>8,14</sup> Low levels of POP-1/Tcf in the nucleus characterize the posterior daughter cells, which assume seam cell fate, while high levels of nuclear POP-1/Tcf characterize anterior daughter cells, which assume hypodermal cell fate.<sup>17</sup> However, the molecular events upstream of POP-1/TCF differ from the canonical Wnt signaling pathway of vertebrates. Unlike the known animal Wnt signaling pathways, which utilize a single form of  $\beta$ -catenin, *C. elegans* Wnt signaling pathways utilize four different  $\beta$ -catenins encoded by *bar-1*, *sys-1*, *wrm-1* and *hmp-2*.<sup>18-20</sup> BAR-1 and SYS-1 bind directly to POP-1 and are termed canonical  $\beta$ -catenins, while WRM-1 and HMP-2, which do not directly bind POP-1, are termed non-canonical  $\beta$ -catenins. However, WRM-1, in complex with LIT-1, signals indirectly through POP-1 by regulating its nuclear export.<sup>19,21</sup> The BAR-1/ $\beta$ -catenin dependent Wnt signaling

pathway is similar in molecular components and mechanism to the vertebrate canonical Wnt pathway, and regulates a number of cell fate determination processes during larval development.<sup>7,8</sup> For example, in the V5 seam cell lineage, the anterior daughter cell of one of the L2 divisions gives rise to a sensory structure called a postdereid rather than becoming a hypodermal cell, and this postdereid cell fate is dependent on *bar-1*/ $\beta$ -catenin.<sup>22</sup> The *C. elegans* BAR-1 dependent canonical Wnt signaling pathway appears to function primarily in cell fate specification that does not involve asymmetric division, which is instead controlled by a non-canonical Wnt signaling pathway that utilizes the WRM-1 and SYS-1  $\beta$ -catenins.<sup>8</sup> In T seam cells, this 'Wnt/ $\beta$ -catenin asymmetry pathway' regulates seam versus hypodermal cell fate by controlling the ratio of nuclear POP-1 to SYS-1 (Fig. 9A).<sup>8</sup> In the anterior cells, which will become hypodermis, nuclear export of WRM-1 and LIT-1 results in high nuclear POP-1 levels and a high POP-1 to SYS-1 ratio, enabling POP-1 function as a transcriptional repressor (Fig. 9A). In the posterior cells, which retain seam cell identity, nuclear export of WRM-1 and LIT-1 is inhibited, and activation of the WRM-1/LIT-1 complex leads to nuclear export of POP-1. The resulting drop in the POP-1 to SYS-1 ratio allows the formation of a POP-1/SYS-1 complex that converts POP-1 from a transcriptional repressor to a transcriptional activator of Wnt signaling pathway genes (Fig. 9A).<sup>8,20,23</sup> The Wnt/ $\beta$ -catenin asymmetry pathway has been modeled from studies in the T seam cells only. Thus, it is not yet known whether this *wrm-1/sys-1* dependent pathway also controls the reiterative asymmetric divisions of the other seam cells, or is a T seam cell-specific mechanism.

The genes of the heterochronic developmental timing pathway control both stage-specific seam cell division patterns as well as loss of seam cell self-renewal capacity in terminal differentiation. A recurring molecular mechanism in the heterochronic pathway is the stage specific expression of microRNAs that downregulate translational expression of target gene products, and thus allows progression to the next developmental stage.<sup>4,13</sup> There are three occurrences of a stage-specific 'microRNA and target' mechanism during larval development: *lin-4* microRNA downregulates *lin-14* and *lin-28* during L1 and L2 stages, the *mir-48*, *mir-84* and *mir-241 let-7* family microRNAs function redundantly to downregulate *hbl-1* during the L2 stage, and *let-7* microRNA downregulates a number of gene transcripts, including *lin-41*, during the L4 stage.<sup>4,13</sup> In each case, loss of function mutation of the microRNA results in retarded seam cell development that involves lack of developmental progression and reiteration of stage specific seam cell division, as well as a delay in or lack of terminal differentiation. For example, triple mutation of the *mir-48*, *mir-84* and *mir-241 let-7* family microRNAs results in L3 reiteration of the L2 stage specific seam cell amplifying divisions and loss of seam cell terminal differentiation.<sup>24</sup> Conversely, loss of function mutation of microRNA target genes results in precocious seam cell development, which involves exit from the self-renewal cell division cycle and premature terminal differentiation. For example, mutation of the *let-7* microRNA target genes *lin-41*, *hbl-1* or *lin-42* results in premature terminal differentiation during the L3 and L4 stages. Thus, the normal function of the L3/L4 stage

heterochronic genes that are microRNA targets appears to be the maintenance of seam cell identity, which includes self-renewal (Fig. 9B). Vertebrate homologs of the *C. elegans* heterochronic genes also regulate stem cell development.<sup>3,4</sup> For example, *lin-28* is one of five factors that work together to induce stem cell pluripotency in differentiated somatic cells.<sup>4,25,26</sup> *lin-28* specifies stem cell pluripotency and self-renewal by inhibiting microRNA processing, including that of *let-7* microRNAs, thus preventing expression of mature microRNAs that induce cellular differentiation.<sup>26-28</sup> *let-7* microRNA target genes have known functions in stem cell regulation. The mouse homolog of LIN-41 (mLIN-41) is a ubiquitin ligase that functions with LIN-28 to suppress *let-7* microRNA activity in stem cells.<sup>29</sup> In another example, both *Drosophila hunchback* and mouse *ikaros*, the homologs of *C. elegans hbl-1*, function to maintain multipotency of neural stem cells.<sup>30,31</sup>

In *Drosophila* and vertebrates, Casein kinase I epsilon and delta isoforms (CKI $\epsilon/\delta$ ) function in cell cycle regulation and act as positive regulators of the canonical  $\beta$ -catenin dependent Wnt signaling pathway.<sup>32</sup> CKI function in the *C. elegans* Wnt signaling pathways has not been definitively determined, but *kin-20*, the sole CKI $\epsilon/\delta$  homolog in *C. elegans*, functions in the seam cell heterochronic pathway.<sup>33</sup> *kin-20* loss of function mutation results in premature seam cell terminal differentiation during the L4 stage, and thus *kin-20/CKI $\epsilon/\delta$*  functions with or in parallel to the L3/L4 stage precocious heterochronic genes, *lin-41*, *lin-42* and *hbl-1* (Fig. 9B). The vertebrate and *Drosophila* homologs of *kin-20* and *lin-42*, CKI $\epsilon/\delta$  and *period* respectively, function together in the broadly conserved circadian timing pathway, and genetic analyses suggest that *kin-20* and *lin-42* may have a similar regulatory relationship in the seam cell heterochronic pathway.<sup>33,34</sup> A further similarity between heterochronic and circadian timing pathway is that the circadian pathway has been shown to regulate the timing of hematopoietic and neural stem cell proliferation and maturation, although the mechanisms of this regulation are not known.<sup>35-37</sup> Since both *kin-20* and *lin-42* are expressed in the seam cells throughout larval development, these CKI $\epsilon/\delta$  and *period* homologs may play a reiterative role in controlling seam cell asymmetric division and self-renewal, either together with or in parallel to the Wnt asymmetry pathway.

Based on the observation that CKI isoforms function in both spatial and temporal patterning pathways to regulate stem cell development, we hypothesized that cross-regulation between the Wnt and heterochronic pathways may be mediated by CKI. To better understand the roles played by CKI in different cell fate determination pathways, we investigated the function of *kin-19/CKI $\alpha$*  during post-embryonic seam stem cell development in *C. elegans*.

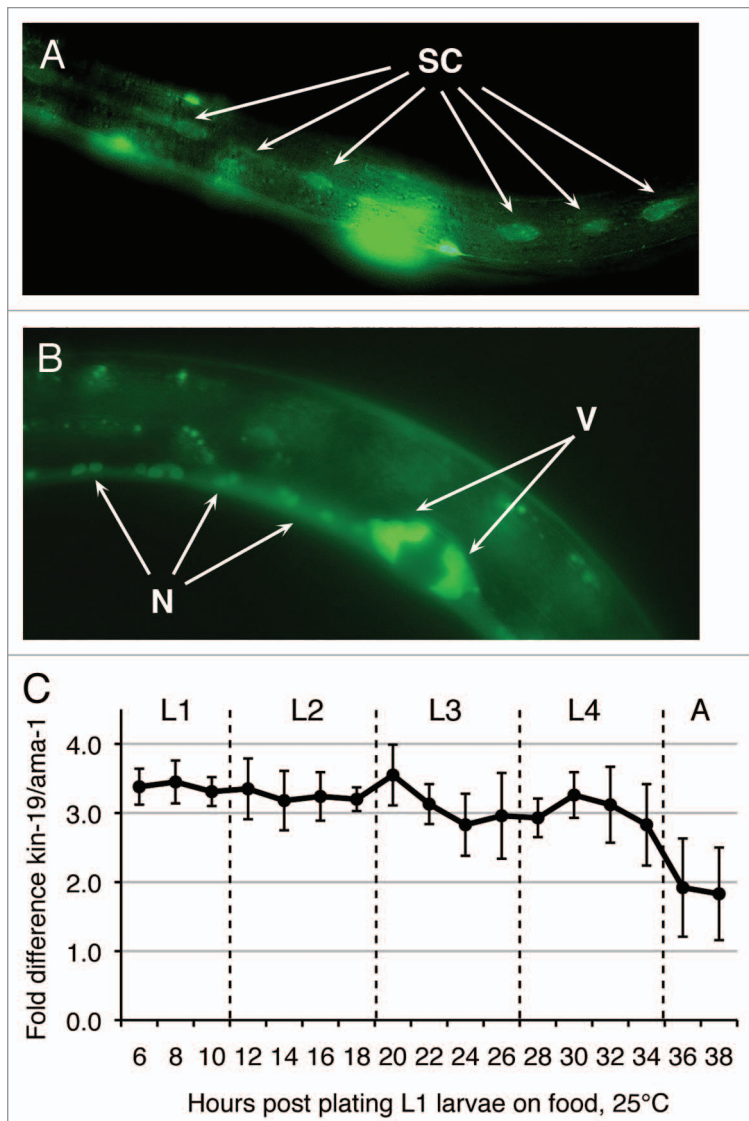
## Results

***kin-19/CKI $\alpha$*  is expressed in the epidermal seam cells throughout larval development.** The casein kinase I (CKI) family in *C. elegans* consists of four genes encoding three different isoforms: *kin-19/CKI $\alpha$* , *kin-20/CKI $\epsilon/\delta$* , *csnk-1/CKI $\gamma$*  and *spe-6/CKI $\gamma$* . We have previously reported that *kin-20/CKI $\epsilon/\delta$*  is expressed in the

seam cells throughout larval development and functions in the heterochronic pathway to regulate seam cell terminal differentiation, while *csnk-1* and *spe-6* either are not expressed, or do not function, in the seam cells.<sup>33</sup> Since both CKI $\alpha$  and CKI $\epsilon/\delta$  are known to function in the vertebrate canonical Wnt signaling pathway, we investigated whether *kin-19/CKI $\alpha$*  is also expressed in the seam cells and/or hypodermis. *Drosophila* and vertebrate CKI $\alpha$  genes encode multiple splice variants that have different functions,<sup>38</sup> but *C. elegans kin-19* is expressed as a single transcript that is expressed at a relatively constant level throughout larval development (Fig. 1). We assayed KIN-19 protein expression using a *kin-19p::kin-19::gfp* translational reporter in wild-type N2 worms, and observed GFP expression in the epidermal seam cells, the vulval cells and in the ventral nerve cord at all larval stages (Fig. 1). We observed KIN-19::GFP expression in both nucleus and cytoplasm of seam cells, and we noticed the formation of a punctate GFP pattern in the cytoplasm. This punctate expression pattern may be indicative of the localization of KIN-19 to cytoplasmic macromolecular complexes, similar to the localization of CKI $\alpha$  to the 'destruction complex' in the canonical Wnt signaling pathway. We did not notice any obvious subcellular asymmetry (nuclear versus cytosolic or anterior versus posterior of single seam cells) or cellular asymmetry (anterior versus posterior daughter cell after mitosis) in KIN-19::GFP expression during the L4 stage.

***kin-19/CKI $\alpha$*  regulates anterior-posterior spatial patterning of seam cell fates.** In the early *C. elegans* embryo, MOM-2/Wnt-signaling polarizes the EMS cell that divides asymmetrically into the E blastomere, which gives rise to endoderm, and the MS blastomere, which gives rise to mesoderm. Disruption of MOM-2/Wnt-signaling results in embryonic lethality due to the transformation of E blastomere to M blastomere, and the production of excess mesoderm at the expense of endoderm.<sup>39</sup> Embryonic lethality due to this 'more mesoderm' (mom) phenotype is also produced by *kin-19* mutation or RNA interference (RNAi), and suggests that *kin-19* is a positive regulator of the asymmetry Wnt signaling pathway in embryos.<sup>40</sup> In order to assess whether *kin-19* plays a similar role during post-embryonic development, we by-passed embryonic lethality by *kin-19(RNAi)* starting in larval stage 1 (L1) animals, and observed the effect on gross development and on epidermal seam cell development. At the gross anatomical level, *kin-19(RNAi)* resulted in adult animals that were thin and elongated in shape, had protruding vulva (Pvl), and were sterile (data not shown). The Pvl phenotype suggested to us that improper specification of vulval cell fates occurs on knockdown of *kin-19* in larval animals, while the elongated body phenotype could have arisen from cell fate transformation that resulted in duplication of the epidermal cell types that determine nematode body length.

For a more detailed analysis, we followed seam cell development using a *C. elegans* strain (*wIs78*) in which the seam cell nuclei and adherens junctions are marked by GFP in wildtype genetic background. We observed *kin-19(RNAi)* animals throughout larval development and detected the formation of excess seam cells during the fourth larval (L4) and adult stages (Figs. 2A and B and 3A and B). In *wIs78* worms, 16 GFP-marked seam cells



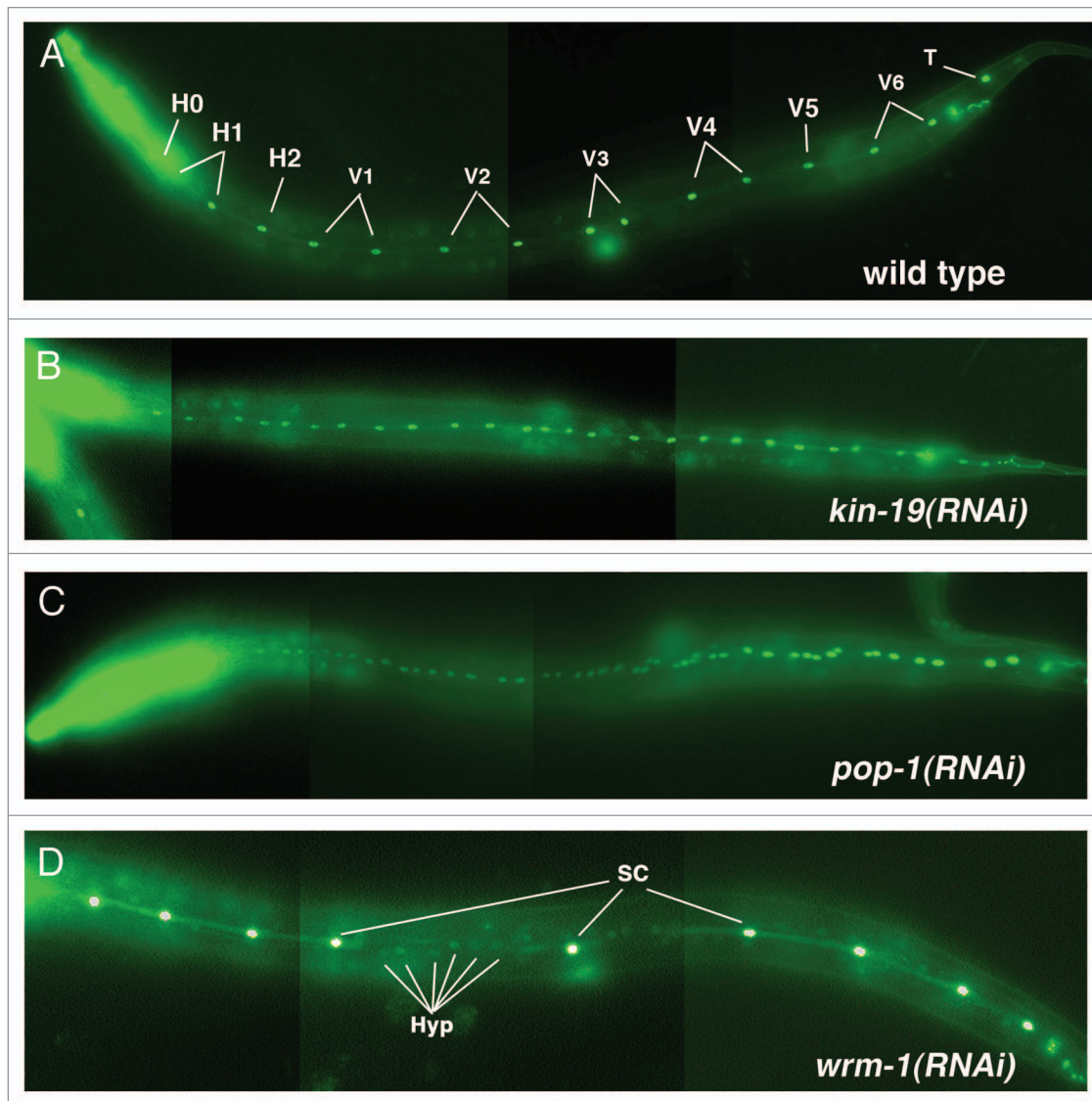
**Figure 1.** *kin-19/CKI $\alpha$*  expression during *C. elegans* larval development. *kin-19::GFP* translational reporter expression in the (A) epidermal seam cells (SC), (B) vulval cells (V) and neuronal cells (N) of the ventral nerve cord in L4 stage wildtype animals. Images taken at 430x. (C) Expression levels of *kin-19* transcript, relative to *ama-1* transcript, during larval stages in wildtype *C. elegans* grown at 25°C.

enter the L4 stage and synchronously go through one round of mitosis during mid-L4 producing 29 cells (Figs. 3A and 4A). The anterior daughter cells migrate away from the seam and differentiate by fusing with the Hyp7 hypodermal syncytium, and thus lose seam cell specific GFP staining. The posterior daughter cells, which retain seam cell identity, terminally differentiate by fusing to form the seam syncytium, and secrete collagenous ridges called alae. Thus, at the end of the L4 stage in *wls78* animals, 16 GFP-marked seam cell nuclei are present within the seam syncytium, whose boundaries are also marked by GFP (Fig. 2A). In *kin-19(RNAi)* animals, the wild type number of seam cells was observed during the early L3 stages, but an average of 19 seam cells, instead of 14, were seen to enter the L4 stage (Fig. 3B).

This number increased to an average of 28 seam cells in the late L4 stage *kin-19(RNAi)* animals and persisted into the adult stage (Figs. 2B and 3B).

The supernumerary seam cells in *kin-19(RNAi)* animals could have resulted from the failure of anterior daughter cells to migrate out of the seam, that is the Hyp7 fated daughters of seam cell division may have undergone an anterior to posterior cell fate transformation and thus aberrantly retained seam cell identity. This type of hypodermal-to-seam cell fate would result in 27 adult seam cells instead of 14 (Fig. 4B). Indeed, we observed that an average of 28 GFP-marked cells persisted from the mid L4 into the adult stage, and that they behaved like normal seam cells, that is they fused during the late L4 and secreted alae during the early adult stage. However, since we occasionally observed *kin-19(RNAi)* worms with more than 28 seam cell nuclei (up to a maximum of 34 seam cell nuclei), a few of the seam cell must have divided again after the mid-L4 division. An alternative explanation to seam cell fate transformation could be that the normal developmental timing program was retarded in *kin-19(RNAi)* worms, such that the seam cells divided a second time immediately after the mid-L4 division instead of undergoing terminal differentiation. A second round of synchronized seam cell division in the mid L4 stage would result in approximately 40 GFP-marked nuclei being visible in the mid to late L4 stages, and retardation of terminal differentiation would also result in lack of seam cell fusion and lack of adult alae secretion. We observed a maximum of only 34 seam cell nuclei in *kin-19(RNAi)* animals (Figs. 3B and 5A). Moreover, seam cell fusion and alae secretion in *kin-19(RNAi)* animals took place at the appropriate time, during the late L4 transition to young adult (Figs. 7A and 8). Therefore, the extra seam cells in *kin-19(RNAi)* animals most likely did not arise from an extra round of synchronized cell division in the L4 stage, and *kin-19(RNAi)* does not result in a retarded developmental timing defect in seam cell L4 division or terminal differentiation. Instead, our observations are consistent with the hypothesis that *kin-19(RNAi)* causes cell fate transformation from anterior hypodermal fate to posterior seam cell fate during post-embryonic development (Fig. 4B), similar to alteration of endodermal to mesodermal fate on *kin-19(RNAi)* during early embryonic development.

**A non-canonical Wnt/ $\beta$ -catenin pathway dependent on *wrm-1* and *sys-1*  $\beta$ -catenins controls seam cell asymmetric division.** Seam cell fate specification is correlated with asymmetric nuclear distribution of POP-1/Tcf between anterior and posterior post-mitotic daughter cells: POP-1 accumulation in the nucleus of the anterior daughter cells confers non-seam cell/hypodermal identity, while lower levels of nuclear POP-1 in the posterior daughter cells specify seam cell fate<sup>17</sup> (Fig. 9). Thus, we would predict that net reduction of POP-1 in all seam cell progeny would cause cells that were normally fated for hypodermis to be transformed to the seam cell fate. Indeed, we observed

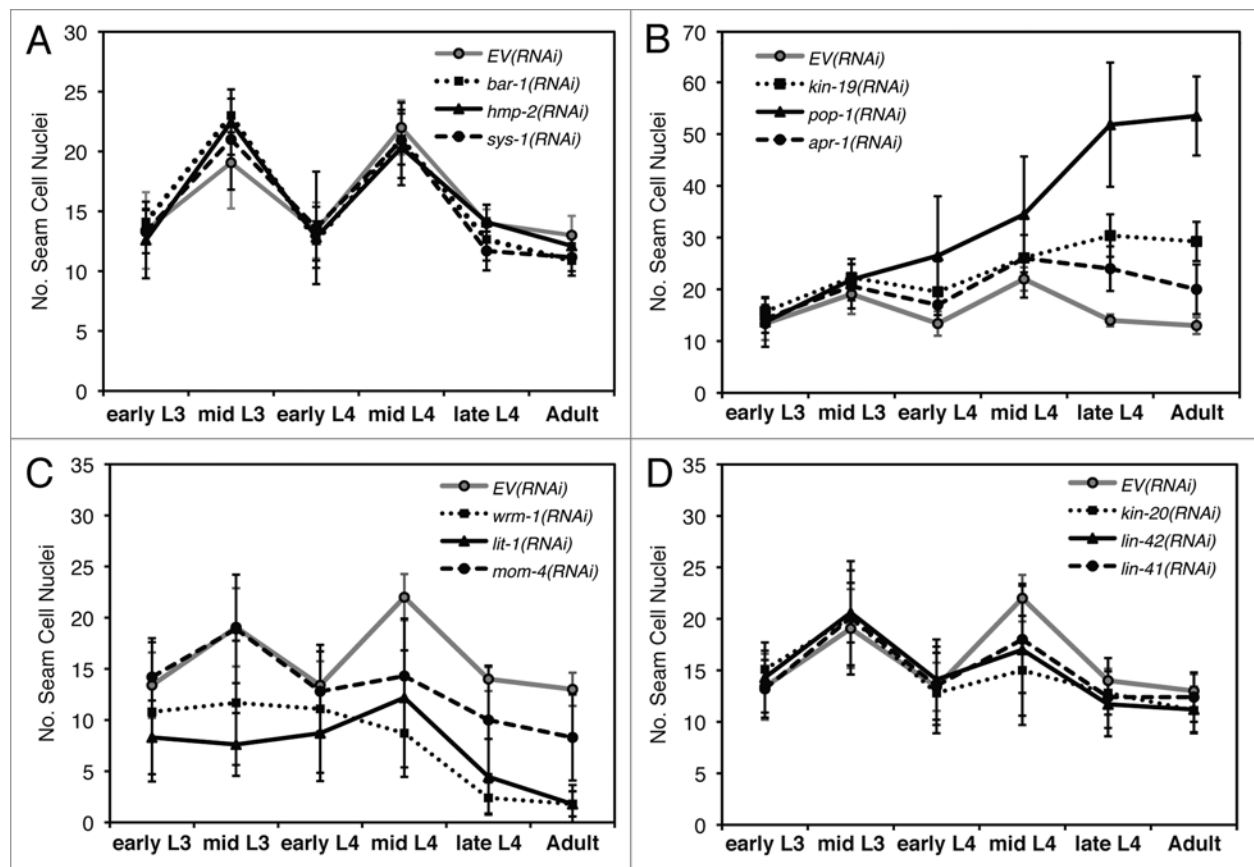


**Figure 2.** Anomalous terminal seam cell numbers result from RNAi knockdown of *kin-9*, *pop-1* or *wrm-1*. (A–D) Young adult or L4 *wls78* worms, in which GFP expression marks the seam cell nuclei (SCM::GFP) and adherens junctions (*ajm-1::gfp*/MH27::GFP). Images are composites of 3 to 4 photographs of the same animal in each case, taken at a magnification of 200x. (A) Young adult *wls78* animal on mock RNAi showing 14 seam cell nuclei within the continuous syncytium of the fused seam cells. (B) Young adult *wls78* animal on *kin-19(RNAi)* showing 27 seam cell nuclei. (C) Young adult *wls78* animal on *pop-1(RNAi)* showing 48 seam cell nuclei. (D) Mid L4 stage *wls78* animal on *wrm-1(RNAi)* showing 9 seam cell nuclei (SC) within the discontinuous seam cell syncytium. Also visible in the plane of the seam cells are the nuclei of cells (Hyp) that have not fused with the seam cell syncytium.

excess post-embryonic seam cells formed on RNAi of *pop-1/Tcf*, very similar to the seam cell phenotype of *kin-19(RNAi)* animals (Figs. 2C and 3B). The supernumerary seam cell phenotype of *pop-1(RNAi)* was more pronounced than that of *kin-19(RNAi)*, and an average of 52 seam cell nuclei were observed in the late L4 and adult stages (Figs. 2C and 3B). This doubling of the seam cell nuclei numbers in the mid L4 stage suggests that the seam cells undergo a second synchronous division after the mid-L4 division (Fig. 4C). The observation that an average of 52 seam cell nuclei persisted into the adult stage, instead of decreasing in number, indicates that a hypodermal-to-seam cell fate transformation occurs in *pop-1(RNAi)* animals (Fig. 4C).

As with *kin-19(RNAi)*, *pop-1(RNAi)* did not result in a developmental timing defect as seam cell fusion and alae secretion in these worms occurred with wild type timing (Figs. 7 and 8). The similarity between the *kin-19* and *pop-1* RNAi seam cell phenotypes, together with previous reports that *kin-19* acts in the early embryo Wnt/ $\beta$ -catenin asymmetry pathway<sup>40</sup> and that CKI $\alpha$  functions as a negative regulator of Wnt signaling in vertebrates and invertebrates,<sup>41</sup> suggests that *kin-19/CKI $\alpha$*  functions with *pop-1/TCF* in a Wnt signaling pathway to regulate asymmetric division of post-embryonic seam cells.

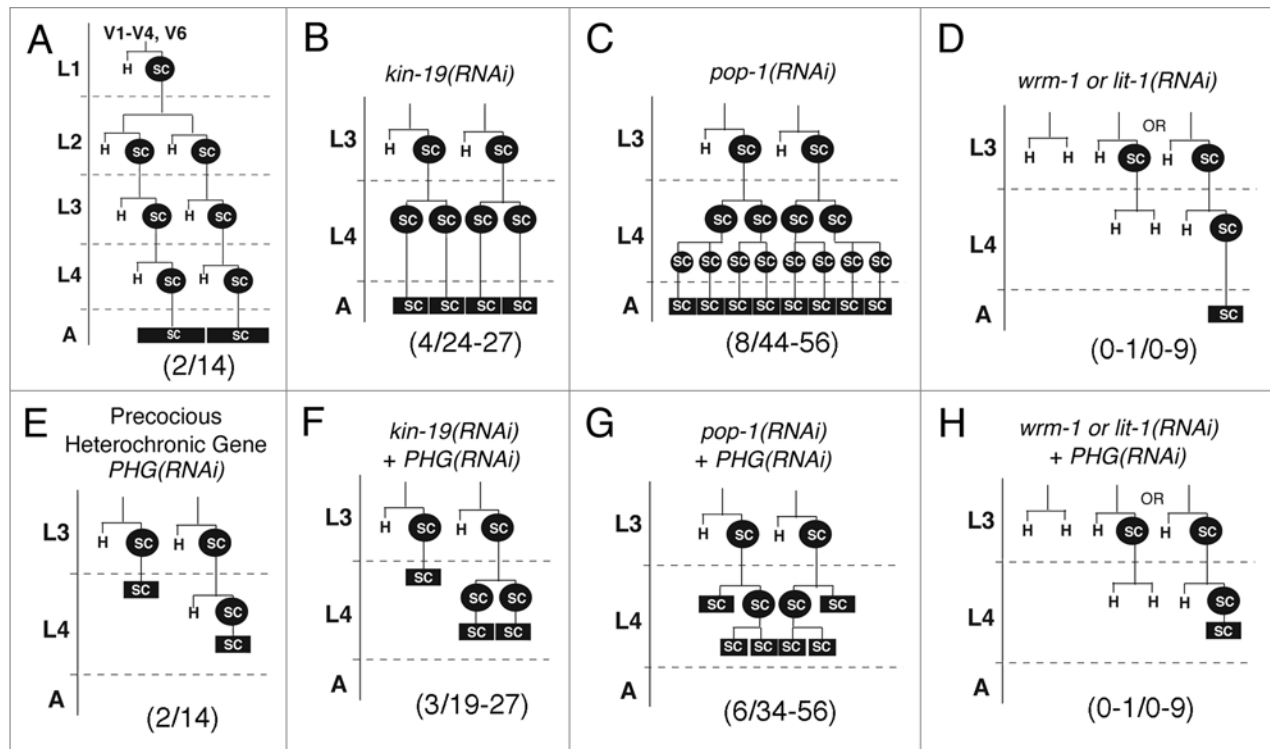
In order to define the Wnt/ $\beta$ -catenin pathway that *kin-19* functions in, we first sought to identify the Wnt signaling pathway



**Figure 3.** (A–H) Anomalous seam cell numbers result from RNAi knockdown of *kin-19* and Wnt signaling pathway genes. For each RNAi condition,  $n = 45$ –50 animals were observed for each time point, and data points are averages of three trials. Error bars indicate standard deviation. (A) *wls78* animals on mock RNAi, and RNAi of *bar-1*, *hmp-2* or *sys-1* showed wildtype seam cell numbers and pattern of division. (B) RNAi knockdown of *kin-19*, *pop-1* or *apr-1* resulted in seam cell hyperplasia starting in the early L4 stage and persisting into the adult stage. (C) RNAi knockdown of *wrm-1*, *lit-1* or *mom-4* resulted in seam cell hypoplasia, starting during the late L2 stage and persisting into the adult stage. (D) RNAi knockdown of the heterochronic genes *kin-20*, *lin-42* or *lin-41* resulted in the normal terminal seam cell number, but fewer than normal seam cells underwent division in the L4 stage. *hbl-1*(RNAi) produced a similar seam cell pattern of division and terminal seam cell number (not shown).

responsible for controlling asymmetric division of V1-4,6 seam cells. Wnt signaling pathways have been shown to regulate asymmetric division of V5.p and T seam cells.<sup>8,14</sup> In the V5.p lineage, a Wnt signaling pathway dependent on *bar-1*/β-catenin is required for the anterior daughter cell of the first L2 division to express postdeireid neural cell fate instead of hypodermal fate, while a Wnt signaling pathway dependent on *wrm-1*/β-catenin regulates asymmetric division of the T seam cell and its progeny in the L1 and L2 stages.<sup>22,42</sup> However, it is not known which β-catenins are involved in regulating non-T and non-V5 seam cell fates. Using post-embryonic RNAi, we observed the effect on seam cell development of knocking-down each of the *C. elegans* β-catenins. RNAi of *bar-1*, *sys-1* or *hmp-2* did not have any discernable effect on V seam cell numbers divisions or terminal seam cell number and terminal differentiation (Fig. 3A). However, we found that knockdown of the non-canonical β-catenin *wrm-1*, or of its associated MAP kinase *lit-1*, resulted in fewer than normal seam cell nuclei (Figs. 2D and 3C). The normal number of seam cells was observed to enter the L2 stage in both *wrm-1*(RNAi) and *lit-1*(RNAi) worms, but the number of GFP-marked cells dropped during the late L2, L3 and L4 stages, and only 1 to 2

seam cells were visible by the late L4 to adult stages. MOM-4/MAPKKK phosphorylates and activates the WRM-1/LIT-1 complex,<sup>43</sup> and *mom-4*(RNAi) also resulted in loss of seam cells, although the phenotype was less pronounced and seam cell loss was not observed until the L4 stage (Fig. 3C). These phenotypes suggest that there was transformation of cell fate from posterior seam cell identity to anterior Hyp7 identity in *wrm-1*(RNAi), *lit-1*(RNAi) and *mom-4*(RNAi) worms (Fig. 4D). In *lit-1*(RNAi) animals, the seam cell number increased modestly during the mid-L4 indicating that some of the seam cells had divided, but the observation that the final seam cell number dropped down to approximately two cells suggests that nearly all the posterior daughter cells of seam cell division underwent fate transformation to hypodermal fate (Fig. 4D). This anteriorization of cell fate is opposite to the phenotype seen on RNAi of either *kin-19* or *pop-1*, and is consistent with the current model of the WRM-1/SYS-1 dependent Wnt/β-catenin asymmetry pathway proposed for T seam cells (Fig. 9).<sup>8</sup> According to the current model, in the posterior post-mitotic daughter cell WRM-1, LIT-1 and MOM-4 function together to phosphorylate POP-1 and induce its nuclear export, thus reducing the POP-1 to SYS-1 nuclear ratio and

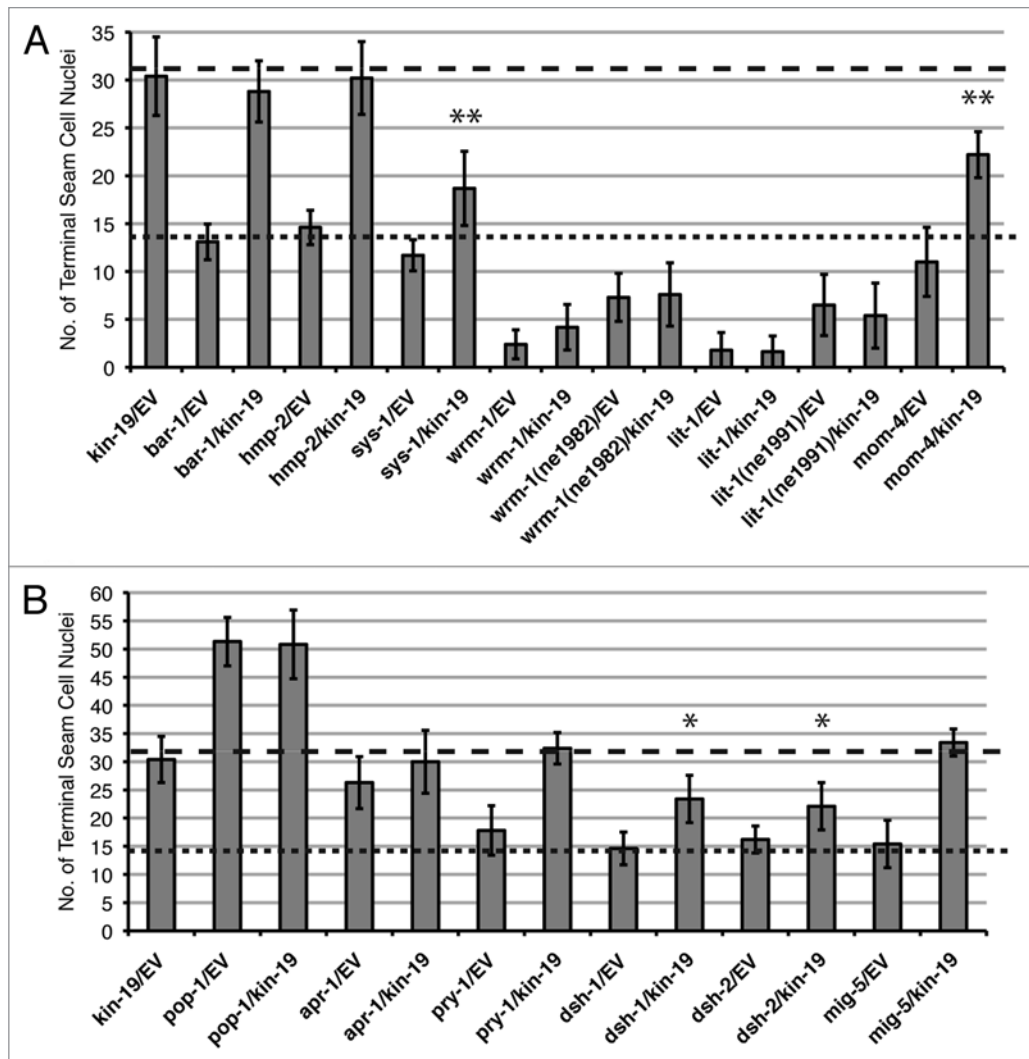


**Figure 4.** Post-embryonic lineages of epidermal seam cells V1-4 and V6 on RNAi knockdown of *wnt* signaling pathway genes and/or heterochronic genes. H: hypodermal cell, SC: seam cell, SC boxed in black indicates terminally differentiated seam cells that have fused into a syncytium. L1-L4: larval stages 1 to 4, A: adult. Numbers in parentheses at the bottom of each lineage indicate the number of terminal seam cells within the lineage shown (first number), and the range of terminal seam cells that results if all V-seam cells undergo the lineage shown (second number). (A) Normal pattern of seam cell (V1-4, 6) division and differentiation during larval development. (2/14) Each of the V1-4, V6 lineages produce 2 terminal seam cells, and the total number of visible terminal seam cells is 14. (B–D) Models of misspecification of V seam cell lineages in worms on (B) *kin-19(RNAi)*, (C) *pop-1(RNAi)* and (D) *wrm-1(RNAi)* or *lit-1(RNAi)*. (E–H) Models of misspecification of V seam cell lineages in worms on (E) RNAi of precocious heterochronic genes (*PHG-kin-20*, *lin-42*, *lin-41* or *hbl-1*), and (F–H) simultaneous RNAi of a PHG and a *wnt* signaling pathway gene—(F) *kin-19*, (G) *pop-1*, (H) *wrm-1* or *lit-1*.

allowing the formation of the POP-1/SYS-1 transcriptionally active complex (Fig. 9).<sup>21,44,45</sup> A prediction from this model is that RNAi knockdown of *wrm-1*, *lit-1* or *mom-4* would lead to net increase of nuclear POP-1 in all seam cell progeny, thus prevent the formation of the POP-1/SYS-1 transcriptional complex, and cause cells normally fated for seam cell identity to be transformed to hypodermis—as is observed in our experiments.

To test if *C. elegans* Wnt pathway genes interact in a manner consistent with function of the T seam cell Wnt/ $\beta$ -catenin asymmetry pathway in V seam cells, we assayed the effect of simultaneous or double RNAi (dRNAi) of pairs of Wnt pathway genes on seam cell asymmetric division (Table 1). Like *kin-19(RNAi)*, single RNAi of *pop-1*, *apr-1* or *pry-1* resulted in excess seam cells, albeit to varying degrees, and we thus termed these genes seam cell inducers. Double RNAi of *pop-1* with *pry-1* or *apr-1* resulted in *pop-1(RNAi)* phenotype, while dRNAi of *pry-1* and *apr-1* resulted in *apr-1(RNAi)* phenotype. These genetic interactions indicate that APR-1 may function downstream of PRY-1, and that PRY-1 and APR-1 normally function to increase nuclear POP-1 levels in the anterior daughter cell. Indeed, PRY-1 is localized to the cortex and serves as a scaffold for cortical APR-1, WRM-1 and LIT-1, while APR-1 has been observed to shuttle between the cortex and nucleus to aid in the nuclear export of

WRM-1 and thus inhibit nuclear export of POP-1.<sup>44,46</sup> According to the current model of the Wnt/ $\beta$ -catenin asymmetry pathway, hypodermal fate is determined by high nuclear POP-1 and a high POP-1:SYS-1 nuclear ratio, which can be generated by either increasing the amount of POP-1 or decreasing the amount of SYS-1 in the nucleus. Thus, the model predicts that loss of SYS-1 would result in fate transformation from seam cell to hypodermal fate. We did not observe any effect on seam cell asymmetric division with RNAi of *sys-1* alone (Figs. 3A and 5A and Table 1). However, dRNAi of *sys-1* with *pop-1*, *apr-1* or *pry-1* suppressed the excess seam cell phenotype of the seam cell inducer genes, while dRNAi of *sys-1* with *mom-4* further enhanced the loss of seam cell phenotype of *mom-4(RNAi)*. Both these sets of dRNAi results indicate that *sys-1* normally functions in the V seam cells to positively regulate seam cell fate, and is consistent with SYS-1 function as a transcriptional coactivator of POP-1 in T seam cells. We also found that single RNAi of the Dishevelled homologs *dsh-1* and *dsh-2* did not have any effect on seam cell asymmetric division (Fig. 5B and Table 1). However, dRNAi of these genes in combination with *sys-1(RNAi)* resulted in a small decrease in the terminal number of seam cells, while dRNAi of these genes in combination with *apr-1(RNAi)* resulted in suppression of the excess seam cell phenotype (Table 1). Both these interactions



**Figure 5.** Effect of genetic interaction between *kin-19* and Wnt signaling pathway genes on terminal seam cell number, a measure of asymmetric division. (A and B) The number of terminal seam cell nuclei was observed in *wls78* animals fed on simultaneous RNAi of *kin-19* and the indicated Wnt signaling pathway gene, except for the following in (A): *wrm-1(ne1982ts);wls78* or *lit-1(ne1991ts);wls78* mutant animals were grown at and subjected to RNAi of the indicated Wnt signaling pathway gene at 25°C. Results shown are averages of three trials carried out with  $n=50-65$  animals for each RNAi condition. Error bars indicate standard deviation. (\*\*)  $P < 0.05$  and (\*)  $P < 0.1$ , indicates statistically significant suppression of *kin-19*/EV(RNAi) phenotype, determined using students t-test. The dashed line indicates the average terminal seam cell number on *kin-19*(RNAi). The dotted line indicates the wildtype terminal seam cell number.

indicate transformation of cell fate from seam cell to hypodermal cell, and are consistent with a model in which nuclear SYS-1 levels decrease due to inhibition of nuclear import that is normally mediated via Dishevelled molecules.<sup>47,48</sup> We further determined that neither *bar-1*/ $\beta$ -catenin nor *hmp-2*/ $\beta$ -catenin interact with any of the Wnt asymmetry pathway genes or other known *C. elegans* Wnt pathway genes to regulate seam cell asymmetric division (Table 1). Together, our data indicate that asymmetric division of both V seam cells and the T seam cells is controlled by a non-canonical Wnt/ $\beta$ -catenin that involves WRM-1 and SYS-1  $\beta$ -catenins as well as the atypical MAPK pathway kinases LIT-1 and MOM-4.

**The non-canonical Wnt/ $\beta$ -catenin asymmetry pathway in seam cells may function cell autonomously.** In the canonical model of the Wnt signaling pathway in vertebrates, inhibition of

the ‘destruction complex’, subsequent stabilization and nuclear import of  $\beta$ -catenin and expression of Wnt pathway target genes by the  $\beta$ -catenin/Tcf transcription complex all depend on reception and transduction of WNT signals by WNT receptors. In *C. elegans*, there are 5 known Wnt genes (*cwn-1*, *cwn-2*, *egl-20*, *lin-44*, *mom-2*) and 4 known Wnt receptors (*lin-17*, *lin-18*, *mig-1* and *mom-5*). While the downstream molecules of the Wnt/ $\beta$ -catenin asymmetry pathway are conserved in the EMS blastomere and T seam cell, asymmetric division in the EMS blastomere is signaled through reception of MOM-2/Wnt by MOM-5/Wnt receptor, while asymmetric division in the T seam cell is signaled through reception of LIN-44/Wnt by LIN-17/Wnt receptor.<sup>39,49,50</sup> In both cell types, proteins that localize asymmetrically in the cell cortex of the progenitor cell before mitosis, or in one of the daughter cells after mitosis, are thought to connect the

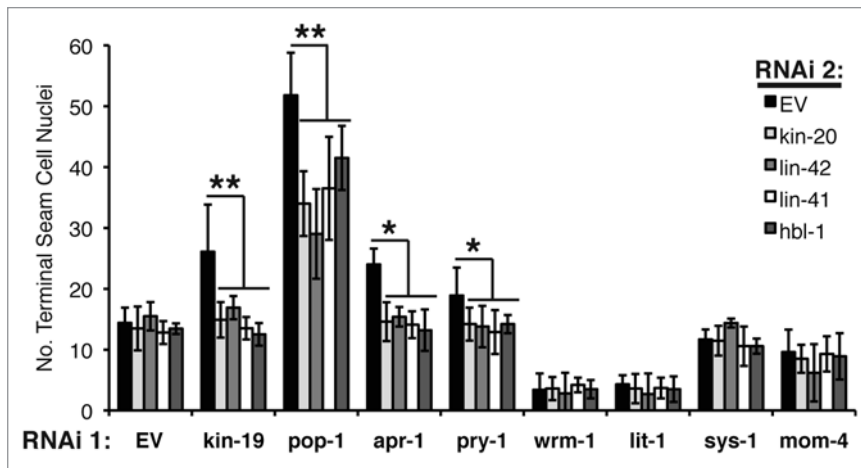
**Table 1.** Terminal seam cell number resulting from genetic interaction between Wnt signaling pathway genes

Wnt Pathway Component	RNAi 1	Terminal Seam Cell Number								
		EV	kin-19	pop-1	apr-1	pry-1	sys-1	wrm-1	lit-1	mom-4
	EV	<b>13±3(+)</b>								
CKIα	kin-19	<b>31±4</b>		<b>51±6</b>	<b>30±6</b>	<b>33±3</b>	<b>18±5</b>	<b>4±3</b>	<b>2±2</b>	<b>22±2</b>
TCF	pop-1	<b>52±4</b>			53±4	51±6	41±4	4±2	3±4	45±3
APC	apr-1	<b>26±3</b>				24±5	20±3	3±1	4±1	20±2
Axin	pry-1	<b>14±2(+)</b>					13±3	2±3	2±3	12±3
β-catenin	sys-1	<b>13±3(+)</b>						2±1	2±2	10±2
β-catenin	wrm-1	<b>2±3</b>							2±1	2±3
MAP kinase	lit-1	<b>2±2</b>								3±2
MAPKKK	mom-4	<b>11±4(+)</b>								
β-catenin	bar-1	(+)	<b>30±6</b>	52±4	26±3	ND	(+)	3±2	4±1	(+)
β-catenin	hmp-2	(+)	<b>33±4</b>	50±7	25±4	ND	(+)	3±3	2±2	(+)
Dishevelled homolog	dsh-1	(+)	<b>27±2</b>	ND	22±3	ND	11±2	ND	ND	(+)
Dishevelled homolog	dsh-2	(+)	<b>25±3</b>	ND	21±4	ND	10±2	ND	ND	(+)
Dishevelled homolog	mig-5	(+)	<b>33±3</b>	ND	24±5	ND	(+)	ND	ND	(+)
GSK3β kinase	gsk-3	(+)	32±4	50±5	24±3	ND	(+)	4±2	2±1	(+)
Wnt receptor	lin-17	(+)	32±3	ND	24±2	ND	10±3	ND	ND	10±1
Wnt receptor	lin-18	(+)	30±5	ND	24±3	ND	10±2	ND	ND	(+)
Wnt receptor	mig-1	(+)	35±2	ND	26±3	ND	(+)	ND	ND	(+)
Wnt receptor	mom-5	(+)	33±3	ND	26±3	ND	11±3	ND	ND	9±3
Wnt	cwn-1	(+)	31±5	ND	25±2	ND	(+)	ND	ND	(+)
Wnt	cwn-2	(+)	30±6	ND	24±3	ND	(+)	ND	ND	(+)
Wnt	egl-20	(+)	29±6	ND	25±3	ND	(+)	ND	ND	(+)
Wnt	lin-44	(+)	34±2	ND	24±4	ND	(+)	ND	ND	(+)
Wnt	mom-2	(+)	30±3	ND	24±2	ND	(+)	ND	ND	(+)
Wnt secretion	mig-14	(+)	30±5	ND	26±2	ND	(+)	ND	ND	(+)

The terminal seam cell number was observed in synchronized populations of young adult *wls78* animals that had been fed on simultaneous RNAi of two Wnt signaling pathway genes. Numbers are averages of 3 independent trials with 25 to 30 animals for each RNAi condition. (+) indicates wildtype terminal seam cell number. EV: empty vector. ND: not determined. Seam cell numbers in bold, enlarged font are also represented in Figure 5.

Wnt signal received by the receptor to downstream transcriptional regulation mediated by POP-1. Specifically, Wnt signaling in the T seam cell asymmetry pathway has been postulated to activate Dsh-homolog mediated nuclear import of SYS-1, and inhibit PRY-1 and APR-1 mediated export and cortical localization of WRM-1, thus in effect decreasing the nuclear POP-1:SYS-1 ratio and allowing transcription by the POP-1/SYS-1 complex (Fig. 9).<sup>20,44-46</sup> In order to determine which Wnts and Wnt receptors regulate the Wnt/β-catenin asymmetry pathway in the V seam cells, we assayed the effect of single and double RNAi of Wnt, Wnt receptor and Wnt signaling pathway genes on seam cell asymmetric division. In addition, we tested the effect of RNAi of *mig-14*, which is expressed in Wnt secreting cells.<sup>51</sup> On single RNAi in *wls78* animals, none of the Wnts, Wnt receptors or *mig-14* affected seam cell asymmetric division (Table 1). Double RNAi with various combinations of the Wnt and Wnt receptor genes also had no effect on normal seam cell asymmetric division (not shown). On double RNAi with *sys-1*, RNAi of the Wnt receptors genes *lin-17*, *lin-18* and *mom-5* resulted in a modest reduction in terminal seam cell numbers, while double RNAi of the same Wnt receptors genes with *mom-4* weakly enhanced the loss of seam cell phenotype (significance  $0.15 < p < 0.3$ ). These results together suggest, but do not conclusively demonstrate, that cell extrinsic Wnt signaling via Wnt and Wnt receptors does not regulate the seam cell non-canonical Wnt/β-catenin asymmetry pathway, which may function cell autonomously.

***kin-19/CKIα* functions in the seam cell non-canonical Wnt/β-catenin asymmetry pathway.** In order to determine whether *kin-19* functions with *wrm-1* and *lit-1*, and what the functional genetic position of *kin-19* is relative to these Wnt pathway genes, we performed semi-epistasis experiments using simultaneous RNAi of *kin-19* with each of the Wnt pathway genes (Fig. 5). Consistent with our findings that the canonical β-catenin *bar-1* and the non-canonical β-catenin *hmp-2* do not appear to regulate V seam cell fate, RNAi of either of these genes had no effect on the supernumerary seam cell phenotype of *kin-19(RNAi)*. An evolutionarily conserved feature of the β-catenin dependent canonical Wnt signaling pathway is the dual kinase regulation of β-catenin stability by CKIα and GK3β in the ‘destruction complex’.<sup>2,52</sup> In vertebrates, CKIα phosphorylation of β-catenin is primed by CKIα phosphorylation by GK3β, which also phosphorylates β-catenin. Indeed the conjunction of GK3β and CKI activities has been detected in a number of other pathways, such as the Hedgehog (Hh) pathway in which GK3β and CKI control the stability of Cubitus (Ci) transcription factor in response to Hh signaling, and the Drosophila and vertebrate circadian pathways.<sup>53,54</sup> However, *gsk-3(RNAi)* resulted in the normal terminal seam cell number, and dRNAi of *kin-19* and *gsk-3* resulted in the *kin-19(RNAi)* phenotype (Table 1). Similarly, dRNAi of *gsk-3* with other genes of the asymmetric Wnt pathway did not have any effect on seam cell development beyond the phenotypes of single Wnt gene RNAi (Table 1). Thus, *gsk-3* does not appear to



**Figure 6.** Effect of genetic interaction between *kin-19* and heterochronic pathway genes on terminal seam cell number, a measure of asymmetric division. The number of terminal seam cell nuclei was observed in *wls78* animals fed on simultaneous RNAi of *kin-19* and the indicated heterochronic pathway gene. Results shown are averages of three trials carried out with  $n = 50$ – $65$  animals for each RNAi condition. Error bars indicate standard deviation. \*\* $p < 0.05$  and \* $p < 0.1$ , indicates statistically significant suppression of *kin-19*/EV(RNAi) phenotype, determined using students t-test.

function, either alone, with *kin-19* or with Wnt signaling pathway genes, to regulate seam cell asymmetric division.

Single RNAi of the *sys-1*/β-catenin alone did not have a discernable effect on seam cell development, but dRNAi of *sys-1* and *kin-19* resulted in partial suppression of the *kin-19*(RNAi) phenotype, suggesting that *sys-1* functions downstream of *kin-19* (Fig. 5A and Table 1). Dishevelled molecules are thought to mediate nuclear import of SYS-1, thus enabling SYS-1 transcriptional function in complex with POP-1.<sup>47,48</sup> Consistent with this function of Dsh, and with the epistatic relationship between *sys-1* and *kin-19*, dRNAi of *dsh-1* or *dsh-2* and *kin-19* resulted in suppression of the *kin-19*(RNAi) phenotype (Fig. 5A). We further found that RNAi of *wrm-1*, *lit-1* or *mom-4* completely or partially suppressed the seam cell fate posteriorization phenotype of *kin-19*(RNAi), indicating that these Wnt pathway genes also function downstream of *kin-19* (Fig. 5A and Table 1). On the other hand, dRNAi of *kin-19* with *pop-1* resulted in the *pop-1*(RNAi) phenotype, dRNAi with *apr-1* resulted in modest enhancement of the *kin-19*(RNAi) phenotype, and dRNAi with *pry-1* resulted in *kin-19*(RNAi) phenotype (Fig. 5B and Table 1). These results suggest that *pop-1* functions downstream of *kin-19*, while *apr-1* functions in parallel with, and *pry-1* upstream of *kin-19*. Simultaneous RNAi of *kin-19* with any of the five Wnt genes, with any of the four Wnt receptor genes, or with *mig-14* resulted in the *kin-19*(RNAi) asymmetric seam cell division phenotype (Fig. 5B and Table 1), suggesting that *kin-19* regulation of seam cell asymmetric division is not dependent on cell extrinsic Wnt signaling. Together, the results from our dRNAi experiments of *kin-19* with Wnt signaling pathway genes indicate that during *C. elegans* post-embryonic development, asymmetric division of seam cells is regulated by *kin-19* function in a non-canonical Wnt/β-catenin asymmetry signaling pathway that involves the *sys-1* canonical β-catenin, the *wrm-1* non-canonical β-catenin,

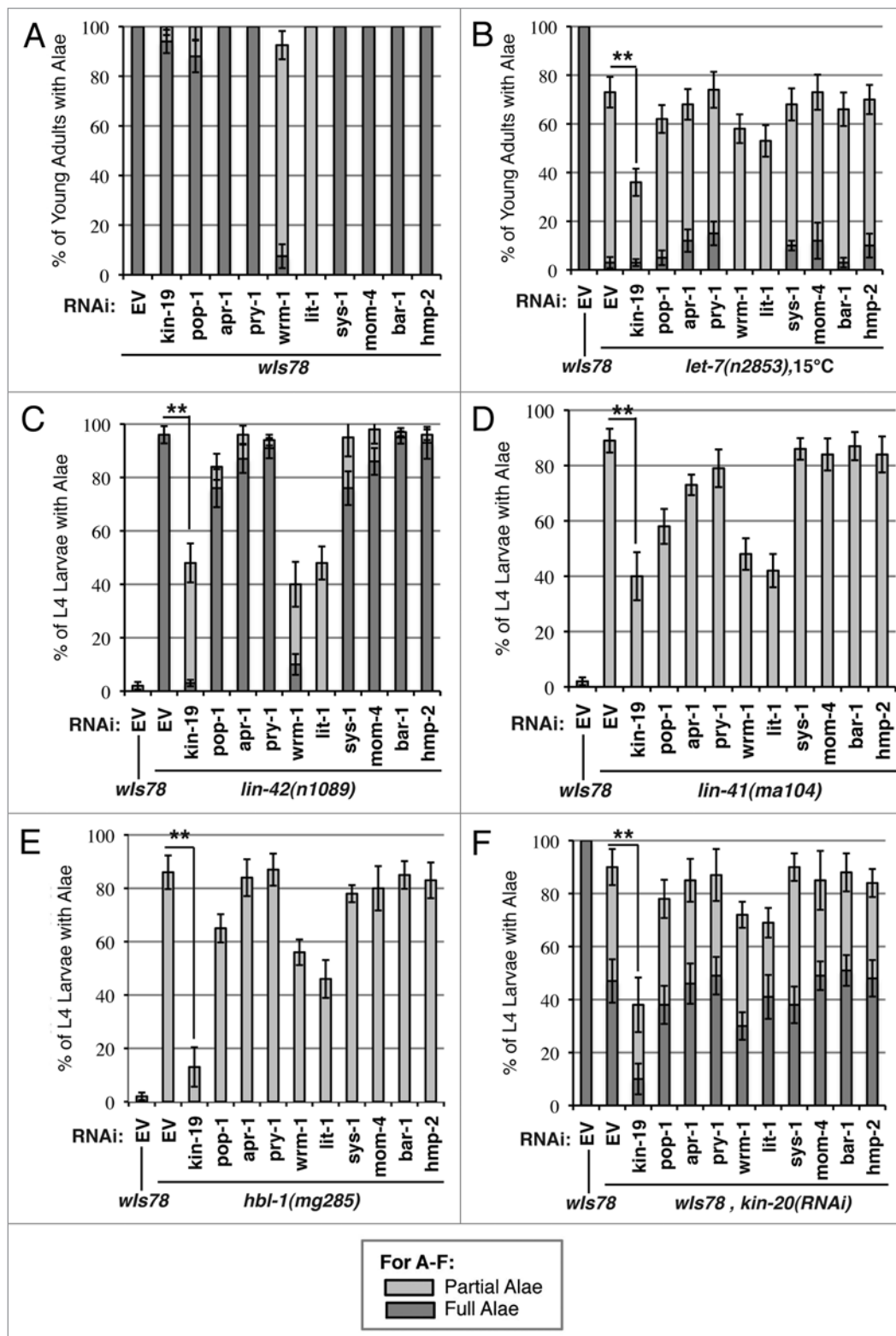
and the *lit-1* and *mom-4* MAP kinases, but does not involve *gsk-3* kinase.

**Heterochronic genes do not regulate seam cell asymmetric division.** *kin-19*/CKIα is closely related to *kin-20*/CKIε/δ, a *C. elegans* heterochronic gene that determines the correct timing of seam cell terminal differentiation.<sup>33</sup> In vertebrate and invertebrate cells, CKIε and CKIα can work additively or in opposition to regulate Wnt signaling pathways.<sup>40,55,56</sup> Thus, we investigated whether *kin-20*/CKIε, or other heterochronic genes, interacts with *kin-19*/CKI, or other Wnt pathway genes, to regulate seam cell asymmetric division. Since the effects of Wnt pathway gene RNAi on seam cell division are mainly observed during late larval development, we focused our analysis on the late larval heterochronic genes, which function during the L3 and L4 stages.

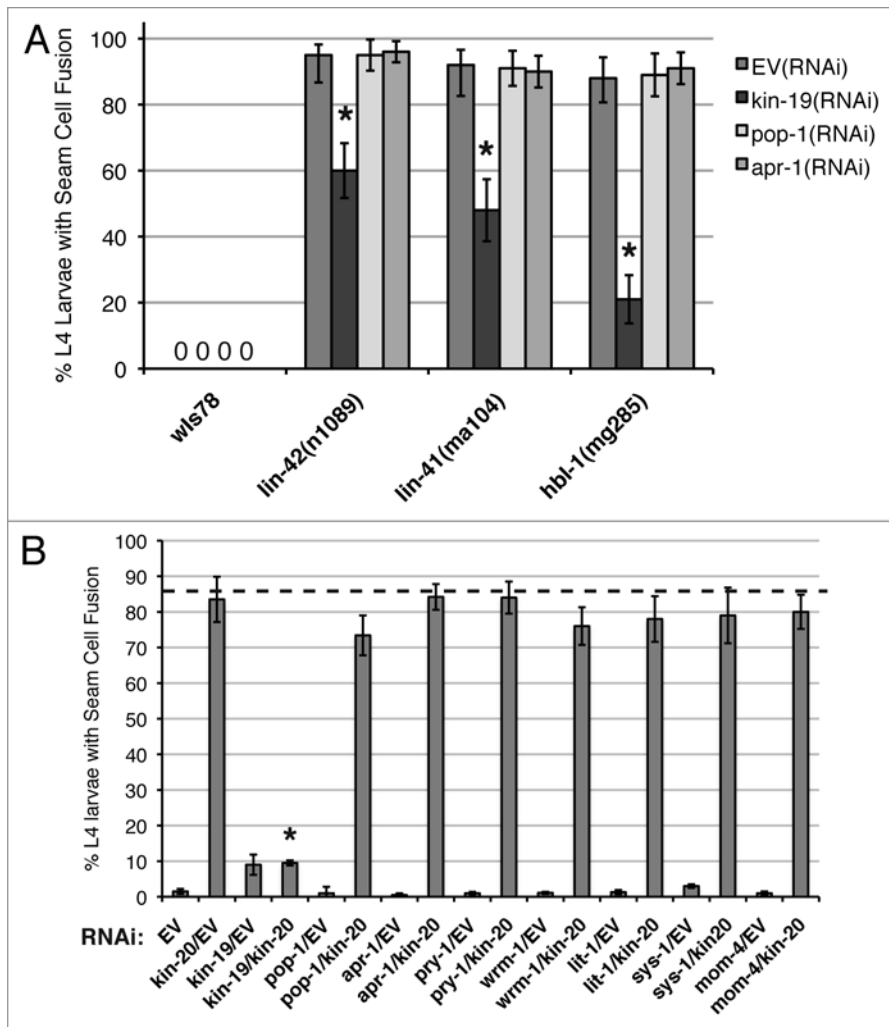
The late larval heterochronic genes regulate seam cell terminal differentiation, which occurs during the L4 to adult transition and consists of exit from the cell cycle and fusion

of the seam cells into a syncytium, followed by secretion of collagenous ridges called alae. *let-7* microRNA functions to allow the acquisition of adult seam cell fates after the L4 stage, and thus *let-7* loss-of-function mutants have retarded terminal differentiation and show delayed and incomplete alae formation as adult animals.<sup>57</sup> *lin-41*, *bbl-1*, *lin-42* and *kin-20* function downstream of, or in parallel to, *let-7* to specify larval seam cell identity and prevent terminal differentiation. Thus, these ‘precocious’ heterochronic genes need to be downregulated at the end of L4 to allow transition to adult fates, and loss of function of any of these *let-7* target genes results in precocious terminal differentiation during the L3 or L4 stage (Fig. 4E).<sup>58–61</sup> One of the functions of the L4 ‘precocious’ genes appears to be inhibition of *lin-29*, whose function is required for terminal differentiation.<sup>62</sup> Thus, loss of function of the ‘precocious’ L4 genes allows LIN-29 function that results in terminal differentiation while loss of function of *lin-29* prevents terminal differentiation, and results in incomplete seam cell fusion and alae secretion.

Single RNAi of the late larval heterochronic genes did not affect terminal seam cell number suggesting that these heterochronic genes do not regulate seam cell asymmetric division (Fig. 6). However, dRNAi of a heterochronic gene with a Wnt asymmetry pathway gene resulted in suppression of the terminal seam cell number phenotype produced on RNAi of *pop-1*, *kin-19*, *apr-1* or *pry-1* (Fig. 6). This result suggests that seam cell hyperplasia, resulting from disruption of asymmetric division and fate specification, depends on function of the ‘precocious’ L4 heterochronic genes. However, a model in which the function of the Wnt asymmetry pathway depends on that of the heterochronic pathway predicts that loss of function of heterochronic genes would affect seam cell asymmetrical division, which is not observed. An alternate and simpler explanation becomes apparent when changes in seam cell numbers are followed over



**Figure 7.** Effect of genetic interaction between *kin-19* and heterochronic pathway genes on alae formation, a measure of terminal differentiation. The extent of alae expression was observed in young adult *wls78* (A) or *let-7(n2853)* (B) worms on RNAi of the indicated Wnt signaling pathways genes. The extent of alae expression was observed in early L4 stage *lin-42(n1089)* (C), *lin-41(ma104)* (D) *hbl-1(mg285)* (E) and *wls78* (F) animals on RNAi of the indicated Wnt signaling pathways genes or double RNAi with *kin-20* (F). Results shown are the average of three trials of each experiment carried out with  $n = 30-40$  animals for each RNAi condition. Error bars indicate standard deviation. \*\* $p < 0.05$  and \* $p < 0.1$ , indicates statistically significant suppression compared to *EV(RNAi)*, determined using students t-test.



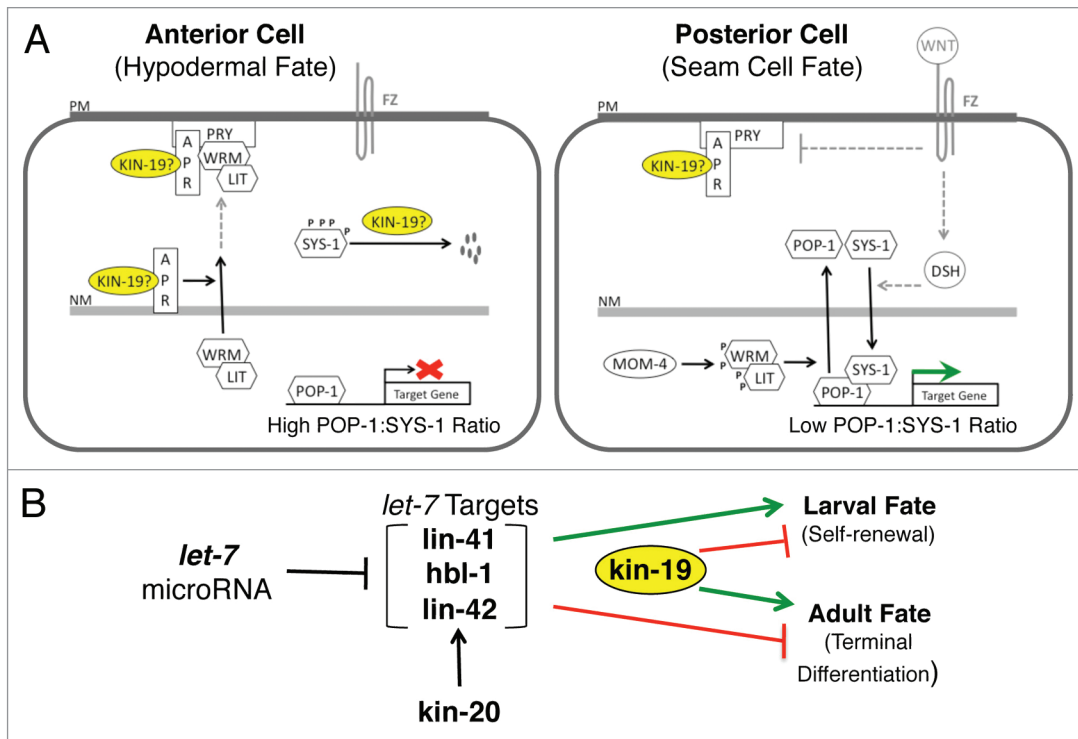
**Figure 8.** Effect of genetic interaction between *kin-19* and heterochronic pathway genes on seam cell fusion, a measure of terminal differentiation. (A) Seam cell fusion in early and mid-L4 animals of the following strains: *wls78*, *lin-42(n1089)*, *lin-42(ma104)*, *hbl-1(mg285)* on RNAi of the indicated Wnt asymmetry pathway genes. (B) Seam cell fusion in early and mid-L4 *wls78* animals on simultaneous RNAi of *kin-20* and the indicated Wnt asymmetry pathway gene. For (B), the dashed line indicates the average number of L4 stage *wls78* animals on *kin-20*(RNAi) showing precocious seam cell fusion. Error bars indicate standard deviation. \*\* $p < 0.05$  and \* $p < 0.1$ , indicates statistically significant suppression compared to *EV*(RNAi), determined using students t-test.

larval development instead of assaying only terminal seam cell number (Fig. 3D). RNAi mediated reduction of function of the precocious heterochronic genes led to a decrease in the maximum number of seam cell nuclei observed during the L4 stage, although initial seam cell number entering the L4 and the terminal seam cell were wild-type (Fig. 3D). The decrease in maximum seam cell number indicates that fewer seam cells undergo division, probably due to seam cells that had precociously terminally differentiated and thus had exited the cell cycle. We have previously reported this phenotype (decrease in maximum number of seam cells) for *kin-20* and *lin-42* loss or reduction of function.<sup>33</sup> Therefore, it is likely that the suppression of seam cell hyperplasia, seen on dRNAi of precocious heterochronic genes with Wnt asymmetry pathway genes, occurs because some seam

cells terminally differentiate during the early L4, resulting in net reduction of seam cells that are capable of undergoing division (Fig. 4E–H). If the Wnt asymmetry pathway regulating seam cell specification was dependent on heterochronic gene function (as suggested by suppression of seam cell hyperplasia), one might also expect an enhancement of the seam cell hypoplasia phenotype. However, we did not observe an effect on seam cell hypoplasia in dRNAi experiments between the precocious heterochronic genes and Wnt asymmetry pathway genes, *wrm-1*, *lit-1*, *sys-1* or *mom-4* (Fig. 6). Finally, we also found that loss of function of the heterochronic genes *let-7* or *lin-29*, either alone or in dRNAi with Wnt asymmetry pathway genes, did not affect seam cell asymmetric division as measured by terminal seam cell number (not shown). Therefore, our data indicate that heterochronic genes do not regulate seam cell asymmetric division, at least in the L4 stage.

**Wnt asymmetry pathway genes do not regulate seam cell terminal differentiation.** Seam cell terminal differentiation involves permanent withdrawal from the cell cycle and loss of self-renewal capacity. *pop-1*(RNAi) and *kin-19*(RNAi) result in dramatic seam cell hyperplasia that involves extra cell divisions, and suggests that seam cell self-renewal capacity is extended, and terminal differentiation prevented, in these animals. We tested whether the Wnt asymmetry pathway genes play a role in regulating seam cell terminal differentiation by assaying the effect of Wnt pathway gene RNAi on seam cell fusion and adult alae expression (Figs. 7 and 8). Single RNAi of *apr-1*, *pry-1*, *sys-1*, *mom-4*, *bar-1* and *hmp-2* resulted in wildtype seam cell terminal differentiation (Figs. 7A and 8). However,

RNAi of *kin-19* or *pop-1* and *wrm-1* or *lit-1* resulted in retarded development of adult alae (Fig. 7A), although seam cell fusion occurred with normal timing (not shown). Since *kin-19* or *pop-1* have the opposite effect on seam cell asymmetric division (RNAi produces seam cell hyperplasia) compared to *wrm-1* or *lit-1* (RNAi produces seam cell hypoplasia), it is difficult to propose a mechanism by which both pairs of genes cause a retarded heterochronic phenotype. However, the observation that the timing of seam cell fusion, which precedes alae secretion, occurs with normal timing in animals on *kin-19*, *pop-1*, *wrm-1* or *lit-1* RNAi argues that adult alae formation is not strictly coupled to seam cell fusion, and that the lack of adult alae in these animals is not a true heterochronic phenotype. In *pop-1*(RNAi) and *kin-19*(RNAi) animals, the retarded alae formation phenotype is modest, and



**Figure 9.** Models of *kin-19* function in *C. elegans* seam stem cells. (A) A simplified model of differential fate specification in daughter cells mediated by the Wnt/ $\beta$ -catenin asymmetry pathway, and the possible roles of KIN-19 in this pathway. FZ: Frizzled, Wnt receptor, PM: plasma membrane, NM: nuclear membrane, P: phosphorylation. Pathway components that do not appear to function in the V seam cell asymmetry pathway (Wnt, FZ) are shown in grey. Indirect and speculative interactions are indicated by dashed, grey lines. (B) The relationship between *kin-19* and the late larval heterochronic genes in regulating seam cell terminal differentiation. *kin-19* likely functions downstream of and in parallel to the heterochronic genes to act as a positive regulator of terminal differentiation.

the areas along the seam that lack alae correlate well with areas where seam cell hyperplasia is most severe. However, in all cases that we observed, the seam cells had laterally fused into a syncytium by the late L4 and young adult stage, and we did not observe division of seam cell nuclei after syncytium formation. Therefore, the mis-specified extra seam cells in *kin-19(RNAi)* and *pop-1(RNAi)* animals fuse to form the seam syncytium at the normal developmental time, and the extra seam cell nuclei within the syncytia do not undergo nuclear division after syncytium formation. The reduction in adult alae in these animals may simply reflect a loss of competence of the seam cells to activate alae synthesis pathways, even though the seam cells have fused. In *wrm-1(RNAi)* and *lit-1(RNAi)* animals, the retarded alae formation phenotype is severe (Fig. 7A), and the extent of lack of adult alae correlates with the loss of seam cells due to misspecification as hypodermal cells. We also observed that contiguous seam cells in these RNAi animals fused into mini-syncytia and secreted alae at the normal developmental time. Therefore the severe, seemingly retarded heterochronic phenotype of lack of adult alae is a consequence of misregulated seam cell asymmetric division and cell fate misspecification, and not due to delayed seam cell terminal differentiation.

We assayed for enhancement of the retarded terminal differentiation phenotypes of *let-7(n2853)* mutant animals and suppression of the precocious terminal differentiation phenotypes of

*lin-42(n1089)*, *lin-41(ma104)*, *hbl-1(mg285)* and *kin-20(RNAi)* animals. For all heterochronic mutants tested, RNAi of *apr-1*, *pry-1*, *sys-1*, *mom-4*, *bar-1* or *hmp-2* did not significantly alter the heterochronic phenotypes of the mutant or RNAi animals in terms of either seam cell fusion or adult alae expression (Fig. 7B–F). RNAi of *pop-1*, *wrm-1* or *lit-1* enhanced the retarded alae phenotype of the *let-7(n2853)* mutant and suppressed the precocious alae phenotypes of the other heterochronic gene mutants and *kin-20(RNAi)* animals (Fig. 7B–F). However, retardation or enhancement of adult alae formation was not mirrored by similar effects on seam cell fusion, which occurred with normal developmental timing in all cases (Fig. 8). Therefore, *pop-1*, *wrm-1* and *lit-1* do not affect the timing of seam cell terminal differentiation, as indicated by normal seam cell fusion. The effect of RNAi of these genes on heterochronic adult alae phenotypes can be explained by the inability of excess seam cells in *pop-1(RNAi)* animals to fully undergo terminal differentiation and form adult alae, and by the loss of alae producing seam cells in *wrm-1(RNAi)* and *lit-1(RNAi)* animals (Fig. 4). The results of double RNAi experiments of heterochronic genes with Wnt pathway genes, and RNAi of Wnt pathway genes in heterochronic gene mutants indicate that the Wnt asymmetry pathway genes, as well as *bar-1* and *hmp-2*, do not regulate the timing of seam cell terminal differentiation.

*kin-19* has dual function in Wnt asymmetric pathway and heterochronic pathway. We propose a model in which the Wnt asymmetry genes and the L4 heterochronic genes function in separate and parallel pathways to control seam cell asymmetric division and terminal differentiation, respectively. RNAi phenotypes of seam cell development for all of the Wnt pathway genes and heterochronic genes tested are consistent with this 'separate and parallel' model, with the notable exception of *kin-19*. Among the Wnt asymmetry pathway genes, only RNAi of *kin-19*, in combination with loss of function of the L4 heterochronic genes, resulted in suppression of both precocious alae formation and precocious seam cell fusion (Figs. 7 and 8). These results indicate that loss of *kin-19* results in bona fide alteration to the timing of seam cell terminal differentiation. *kin-19(RNAi)* also enhanced the retarded heterochronic phenotype of *let-7(n2853)* mutant animals, and is consistent with *kin-19* function either downstream or parallel to *let-7* and the L4 precocious heterochronic genes (Fig. 9). The results of the dRNAi experiments indicate that *kin-19* normally functions as a positive regulator of seam stem cell terminal differentiation (Fig. 9). However, *kin-19(RNAi)* alone does not produce a discernable heterochronic phenotype. Therefore, the primary function of *kin-19*, as part of the Wnt asymmetry pathway in the seam cells, appears to be the regulation of asymmetric division. But *kin-19* has a secondary function, outside of the Wnt asymmetry pathway and most likely in parallel with the heterochronic pathway, in regulating seam cell terminal differentiation.

## Discussion

We present the first example of a single CKI isoform, CKI $\alpha$ , acting to regulate both asymmetric division and terminal differentiation in the *C. elegans* epidermal stem cells. We report that *kin-19/CKI $\alpha$*  regulates seam stem cell fate based on asymmetric division, and functions in a non-canonical Wnt pathway that involves the  $\beta$ -catenins *wrm-1* and *sys-1*. Furthermore, we show that *kin-19* also interacts with the late larval heterochronic genes to regulate seam stem cell withdrawal from self-renewal and allow terminal differentiation. However, other known components of *C. elegans* canonical and non-canonical Wnt signaling pathways do not show a similar interaction with heterochronic genes and do not appear to play a role in seam cell terminal differentiation. Similarly, we found that the late larval heterochronic genes did not play a role in regulating seam cell asymmetric division. The seam cell Wnt/ $\beta$ -catenin asymmetry pathway and the heterochronic temporal identity pathway thus appear to function separately and in parallel to regulate different aspects of seam stem cell fate. These two pathways appear to have at least one point of molecular intersection at *kin-19/CKI $\alpha$* , whose regulation and function may serve to integrate signaling through the spatial and temporal pathways that regulate seam stem cell development.

**The role of *kin-19/CKI $\alpha$*  in the seam cell Wnt/ $\beta$ -catenin asymmetry pathway.** CKI $\alpha$  is a conserved component of the canonical Wnt/ $\beta$ -catenin signaling pathway in *Drosophila* and vertebrates, and most studies indicate that it functions as a negative regulator of  $\beta$ -catenin stability, and thus as a negative regulator of

Tcf/Lef-mediated transcription of Wnt pathway target genes.<sup>16,63</sup> Asymmetric division in the post-embryonic seam cells is controlled by a non-canonical Wnt/ $\beta$ -catenin pathway that utilizes  $\beta$ -catenin nuclear localization instead of  $\beta$ -catenin stability (Fig. 9). Therefore, a direct comparison between the canonical Wnt/ $\beta$ -catenin and non-canonical Wnt/ $\beta$ -catenin asymmetry pathways is inappropriate. However, our studies indicate that *kin-19/CKI $\alpha$*  normally functions as a negative regulator of nuclear localization, or nuclear activity, of both *wrm-1/ $\beta$ -catenin* and *sys-1/ $\beta$ -catenin* in the seam cells. Downregulation of *wrm-1* or *sys-1* function has the opposite effect on seam cell specification (seam cell hypoplasia) compared to downregulation of *pop-1* or *kin-19* function (seam cell hyperplasia) (Figs. 2, 3 and 5A). Additionally, semi-epistasis analysis by double RNAi suggests that *wrm-1* and *sys-1* function downstream of *kin-19* (Fig. 5A and Table 1). Thus, *kin-19/CKI $\alpha$*  normally functions in the postembryonic seam cells to decrease the POP-1:SYS-1 ratio and thus activate the POP-1:SYS-1 transcriptional complex. There appears to be developmental and/or cell specific variations in *kin-19/CKI $\alpha$*  function. In contrast to its post-embryonic role, *kin-19/CKI $\alpha$*  functions as a positive regulator of the Wnt/ $\beta$ -catenin asymmetry pathway during asymmetric division of the EMS blastomere. Loss of function of *kin-19* or of *wrm-1* or *pop-1* results in the same *mom* phenotype, in which excess mesoderm is specified at the expense of endoderm.<sup>39</sup> These differences in embryonic versus post-embryonic function may be due to differences in the upstream signaling cascades that converge on WRM-1 and SYS-1  $\beta$ -catenins and POP-1. In the EMS blastomere, the asymmetry pathway is signaled by cell extrinsic MOM-2/Wnt, which is detected by the transmembrane MOM-5/Wnt receptor, and the signal is transduced through DSH-2 and MIG-5 Dishevelled molecules and GSK-3 $\beta$  kinase.<sup>39,64,65</sup> In contrast, we find that GSK-3 $\beta$ , Dsh molecules, Wnt receptors and Wnt molecules do not appear to have a function in the post-embryonic seam cell Wnt asymmetry pathway (Table 1). Indeed, the V seam cell Wnt asymmetry pathway may be regulated differently from the analogous pathway in T-seam cells, in which asymmetric division is signaled by cell extrinsic LIN-44/Wnt that is received by the transmembrane LIN-17/Wnt receptor.<sup>39,49</sup> Because of functional redundancy among Wnt molecules,<sup>66-68</sup> we cannot unequivocally state from the results of our single RNAi experiments that Wnt signaling does not activate the V seam cell Wnt asymmetry pathway. However, the modest effect or lack of effect on asymmetric division with RNAi of Wnt receptor genes suggests that the V seam cell Wnt asymmetry pathway may function cell autonomously, while asymmetric division of the T seam cell and embryonic EMS blastomere is regulated cell extrinsically by Wnt signaling.

What are the regulatory targets and mechanistic function of KIN-19 in the seam cell Wnt asymmetry pathway? Since *kin-19(RNAi)* results in specification of excess seam cells at the expense of Hyp7 cells, the normal function of KIN-19 must be as a positive regulator of Hyp7 cells, in which the nuclear POP-1:SYS-1 ratio is high, and/or as a negative regulator of seam cells, in which the nuclear POP-1:SYS-1 ratio is low. The simplest model of KIN-19 function is that KIN-19 positively regulates nuclear export of WRM-1, and does so possibly in complex with APR-1 and/or

PRY-1. Thus, *kin-19(RNAi)* would result in increased levels of WRM-1/LIT-1, decreased levels of POP-1, decrease in the POP-1:SYS-1 ratio and specification of seam cell fate—as is experimentally observed (Fig. 9). APR-1 is thought to enable WRM-1 nuclear export and localization to the cell cortex, where it forms a complex with PRY-1.<sup>8,46,69</sup> In our study, *apr-1(RNAi)* resulted in an excess seam cell phenotype that is similar to, although less severe than, *kin-19(RNAi)*, suggesting that APR-1 and KIN-19 regulate the Wnt asymmetry pathway in the same manner (Figs. 3B and 5A). Furthermore, the genetic epistatic relationship between *pry-1* and *kin-19* is the same as that between *pry-1* and *apr-1*, which are known to function in parallel (Fig. 9).<sup>8,46,69</sup> Double RNAi of *apr-1* and *kin-19* did not substantially enhance one or the other phenotype, also suggesting that APR-1 and KIN-19 function in parallel (Fig. 5A). This model of KIN-19 function predicts that KIN-19 should co-localize with APR-1 and/or PRY-1, and that KIN-19 may shuttle between the nucleus and the cell cortex, as has been observed for APR-1.<sup>69</sup> While we have not yet rigorously tested these predictions, we observed that KIN-19::GFP is expressed in both nucleus and cytoplasm. Furthermore, cytoplasmic KIN-19::GFP in seam cells formed a punctate expression pattern, which could indicate the presence of macromolecular complexes, such as that of KIN-19 with APR-1, PRY-1 and other molecules. Additional support for our model of *kin-19* function comes from the fact that the association of CKI $\alpha$  and Axin and APC to regulate  $\beta$ -catenin is a conserved mechanism in the vertebrate and *Drosophila* canonical Wnt pathways.<sup>2,15</sup>

The function of KIN-19 may be to regulate the amount of nuclear SYS-1, in addition to or as alternative to regulating nuclear POP-1 levels via WRM-1 localization. A well-documented function of Casein Kinase I is regulation of a target protein's stability via phosphorylation that leads to Ubiquitin-mediated proteolysis.<sup>16</sup> For example, in the canonical Wnt signaling pathway of vertebrates, CKI $\alpha$  phosphorylation is the key step that results in  $\beta$ -catenin degradation.<sup>16</sup> In the *C. elegans* non-canonical Wnt asymmetry pathway, SYS-1  $\beta$ -catenin is primarily regulated by the protein degradation system.<sup>70</sup> KIN-19 may be localized to the anterior seam daughter cell, which becomes specified as Hyp7, and function to target SYS-1  $\beta$ -catenin for degradation. Therefore, *kin-19(RNAi)* would result in higher nuclear SYS-1 levels, a lower POP-1:SYS-1 nuclear ratio and misspecification of the anterior daughter cell as seam cell. In both models of KIN-19 activity in the seam cell asymmetry pathway, KIN-19 primarily functions in the anterior daughter cell that is fated to become hypodermis (Fig. 9). We did not observe asymmetric localization of KIN-19:GFP during seam cell division, but anterior cell-specific localization of KIN-19 may not be necessary as its postulated interactors, APR-1 and PRY-1, are localized to the anterior cortex before cell division.<sup>8,44</sup>

**The relationship of *kin-19* to the heterochronic genes in regulating seam cell terminal differentiation.** Our studies indicate that *kin-19* has dual and independent function in Wnt/ $\beta$ -catenin asymmetry and heterochronic pathways. While the retarded or precocious terminal differentiation phenotypes, caused by reduction of function of L4 heterochronic genes, are partially dependent on *kin-19/CKI $\alpha$* , other components of the Wnt asymmetry

pathway do not interact genetically with the L4 heterochronic genes (Fig. 7 and 8). Therefore, it appears that *kin-19* does not signal through POP-1 or any of the *C. elegans*  $\beta$ -catenins to exert its regulation of terminal differentiation. Our semi-epistasis RNAi experiments did not involve null alleles and thus it is not possible to accurately define the genetic relationship between *kin-19* and the late larval heterochronic genes. While there are no reports of CKI involvement in microRNA expression, biogenesis or function, in yeast and a number of mammalian cell lines, CKI has been shown to co-localize and function with nuclear RNA processing factors, and also to regulate activity of eIF-4E mRNA cap-binding protein.<sup>71,72</sup> Thus, KIN-19/CKI $\alpha$  may have a function in microRNA processing that has not yet been defined. However, since *kin-19* loss of function alone does not have a discernable heterochronic phenotype, and *kin-19* loss of function phenotype is unaffected by the L4 heterochronic genes (Figs. 6–8), it is more likely that *kin-19* does not function directly in the heterochronic pathway, and instead functions in parallel to the heterochronic genes to control the timing of terminal differentiation (Fig. 9).

**How does KIN-19/CKI $\alpha$  regulate terminal differentiation and loss of self-renewal in seam stem cells?** Seam cell terminal differentiation is characterized by a sequence of cellular events including mitotic cell cycle withdrawal, cell fusion to form a syncytium and the expression of adult seam cell specific genetic programs, such as for alae secretion. CKI $\alpha$  in animals systems other than *C. elegans* is known to regulate cell cycle progression and cell adhesion, as well as regulate the function of transcription factors involved in differentiation.<sup>32</sup> For example, studies in yeast and mammalian cell culture show that CKI $\alpha$  prevents progression from G<sub>2</sub> to mitosis by directly phosphorylating Cdc25 phosphatase, which a key regulator of this transition.<sup>73,74</sup> Therefore, in these systems CKI $\alpha$  acts as a positive regulator of cell cycle withdrawal by preventing cell cycle progression. *C. elegans* has four *cdc-25* genes, two of which have well-defined, but partially redundant, functions in regulating cell cycle progression in embryonic germline stem cells.<sup>75–77</sup> Post-embryonic functions of the *C. elegans cdc-25* genes have not been rigorously tested, and thus it is possible that these genes may function redundantly to regulate cell cycle progression in somatic cells, such as the seam cells. Another point at which KIN-19/CKI $\alpha$  could regulate seam cell withdrawal from mitosis is through the *C. elegans* homologs of the calcium sensor, calmodulin (CaM) and/or FADD (Fas-associated protein with Death Domain). In vertebrates, FADD and Calmodulin act together to regulate cell cycle progression, cell proliferation and apoptosis. Studies in mice and vertebrate cell lines show that CKI $\alpha$  directly phosphorylates FADD and CaM in vitro, and that loss of CKI $\alpha$  phosphorylation function leads to lack of FADD binding to CaM, and results in cell proliferation due to lack of checkpoint arrest at the G<sub>2</sub> to M transition.<sup>78,79</sup> KIN-19/CKI $\alpha$  could similarly interact with the products of the *C. elegans* CaM homologs (*cmd-1*, *cal-1*, *cal-2*, *cal-3*, *cal-4*) and/or with the *C. elegans* FADD homolog. Therefore, *kin-19* loss of function could result in lack of CaM and FADD interaction, loss of normal G<sub>2</sub> to M checkpoint control and thus prevent cell cycle withdrawal and inhibit terminal differentiation.

In *C. elegans*, both CDC-25 and CaM are encoded by multiple genes whose products may function redundantly. This possible functional redundancy in control of mitotic cell cycle progression may be a reason for why *kin-19/CKI $\alpha$*  loss of function only has an effect on terminal differentiation in the sensitized backgrounds of heterochronic gene mutants. A final model for KIN-19 regulation of the seam cell cycle is based on the relationship between CKI $\alpha$  and the p53 tumor suppressor. p53 integrates various environmental signals and responds to cellular stress by inducing cell cycle arrest and apoptosis, thus minimizing genetic instability.<sup>80</sup> In undamaged mammalian cells, CKI $\alpha$  has been found to phosphorylate and stabilize p53.<sup>81,82</sup> Therefore, KIN-19 may also normally function to stabilize CEP-1, the *C. elegans* p53 homolog.<sup>80</sup> Reduction in CEP-1/p53 due to *kin-19* loss of function may result in reduction of cell cycle arrest and thus prevent terminal differentiation.

In addition to, or instead of, controlling cell cycle progression, KIN-19 may regulate key transcription factors that control terminal differentiation programs. In vertebrates, the FoxG1 (Forkhead family) transcriptional repressor functions to maintain neural stem cell identity during forebrain neurogenesis.<sup>83,84</sup> Terminal differentiation of the neural stem cell is triggered by nuclear export of FoxG1, and CKI phosphorylation enables nuclear localization and stabilization of nuclear FoxG1.<sup>85</sup> The heterochronic gene product KIN-20/CKI $\epsilon/\delta$  also functions as a positive regulator of self-renewal and a negative regulator of terminal differentiation in seam cells,<sup>33</sup> and CKI $\epsilon/\delta$  and CKI $\alpha$  play antagonistic roles in the vertebrate canonical Wnt signaling pathway.<sup>32</sup> Therefore, KIN-20/CKI $\epsilon/\delta$  and KIN-19/CKI $\alpha$  may function antagonistically to regulate function of one or more of the 15 *C. elegans* Forkhead transcription factors or other transcription factors, which control self-renewal versus terminal differentiation in the seam cells.

In this study, seam cell terminal differentiation is assayed by seam cell fusion and adult alae expression, and KIN-19/CKI $\alpha$  may directly regulate the process of cell fusion. Vertebrate CKI $\alpha$  has been shown to destabilize adherens junctions and reduce cell-to-cell contacts by phosphorylating E-cadherin.<sup>86</sup> If, KIN-19/CKI $\alpha$  plays a similar role in the seam cells, then loss of *kin-19* function would inhibit seam cell fusion and thus delay terminal differentiation.

In summary, our study indicates that *C. elegans* KIN-19/CKI $\alpha$  functions in a Wnt/ $\beta$ -catenin pathway to regulate seam stem cell asymmetric division, and also has a separate function, in parallel with the heterochronic/temporal identity pathway, in regulating seam stem cell terminal differentiation. The dual but independent roles of KIN-19 suggest that different aspects of seam stem cell development may be coordinately regulated through KIN-19. Distinguishing among the proposed models of KIN-19 action, and defining the exact mode of KIN-19 function in the Wnt/ $\beta$ -catenin asymmetry pathway, as well as in regulating terminal differentiation, will depend on characterizing KIN-19 subcellular localization and identifying direct phosphorylation targets in the seam cells. The conserved function of Wnt/ $\beta$ -catenin and heterochronic pathway molecules and CKI in regulating cellular differentiation in a variety of animal

systems suggests that definition of KIN-19 function and regulation in *C. elegans* seam cell development will illuminate the fundamental process of stem cell development in higher vertebrates. In particular, the study of *kin-19* regulation presents the opportunity to define how multiple aspects of stem cell development may be coordinated through a single molecule.

## Materials and Methods

***C. elegans* strains.** Bristol N2 was used as the wild type strain. Strains JR1000/wIs78 (*ajm-1::gfp/MH27::GFP* and *scm-1::gfp*), JR667/wIs51 (*unc-119(e2498)::Tc1*;wIs51), and PS3729/syIs78 (*unc-119(ed4)*; syIs78[*ajm-1::GFP* + *unc-119(+)*]) were used to visualize seam cell nuclei and seam cell junctions. Additional strains used in this work are listed by chromosome as follows: LGI: *lin-41(ma104)*, LGII: *lin-42(n1089)*, LGIII: *wrm-1(ne1982)*, *lit-1(ne1991)*, LGX: *let-7(n2853)*, *hbl-1(mg285)*. All strains were propagated on NGM plates containing *E. coli* OP50 bacterial lawns. Synchronized starved L1 populations were obtained by hatching eggs, isolated from gravid adults, and starving larvae in M9 medium. All strains were cultured at 20°C, unless otherwise noted.

**Microscopy.** Light and fluorescence microscopy were carried out on Zeiss AxioPlan 2 and Zeiss AxioimagerZ1 microscopes and images were captured and analyzed using Zeiss AxioVision software and Hamatsu cameras.

**RNAi analysis.** RNAi constructs for the following genes were obtained from the Ahringer RNAi library: *bar-1*, *bmp-2*, *sys-1*, *pop-1*, *lit-1*, *gsk-3*, *pry-1*, *apr-1*, *mig-5*, *egl-20*. Additional RNAi constructs were created using the pL4440 vector, and were made for the following genes: *kin-20*, *kin-19*, *wrm-1*, *mom-4*, *dsh-1*, *dsh-2*, *mig-5*, *lin-17*, *lin-18*, *mom-5*, *cun-1*, *cun-2*, *lin-44*, *mom-2*, *mig-14*. The specific gene sequence of all RNAi constructs was sequenced to confirm the identity of the insert. RNAi was induced by feeding, starting with L1 stage starved and synchronized animals. RNAi constructs were expressed from *E. coli* HT115(DE3) bacteria, which were grown on NGM media containing 1 mM IPTG to induce expression from the convergent T7 polymerase promoters on the pL4440 vector. All strains feeding on RNAi bacteria were cultured at 20°C unless otherwise noted. Simultaneous RNAi was carried out by growing the two different RNAi bacterial strains to the same optical density and mixing equal volumes of the cultures before plating onto NGM plates. Mock RNAi consisted of feeding animals on bacteria carrying the empty pL4440 vector (EV). The extent of target gene expression knockdown by RNAi was measured by quantitative real time PCR using SYBR green. Target gene expression level was standardized to *ama-1* transcript levels from the same population, and the ratio of target gene:*ama-1* from RNAi and control populations was compared. RNAi efficiencies ranged from 48% to 83% depending on the RNAi construct.

**Plasmids and molecular analysis.** *kin-19::gfp* transcriptional fusion construct. 4.5 kb of the *kin-19* promoter sequence was PCR amplified from *C. elegans* genomic DNA, 0.9 kb of *kin-19* coding sequence was PCR amplified from N2 cDNA, and *gfp* and *unc-54* 3'UTR sequences were PCR amplified from the

plasmid pPD95.75. The *kin-19* translational reporter was constructed by fusing the three PCR fragments by overlap-extension PCR. Primers used are available upon request. The resulting *kin-19p::kin-19::gfp::unc-54 3'UTR* fragment was injected into N2 animals at 50 ng/μl along with *rol-6* at 75 ng/μl as a coinjection marker. Five transgenic lines were identified by the rolling phenotype caused by *rol-6*. Rolling animals were re-examined to confirm the presence of GFP, and spatial and temporal expression patterns of the GFP were examined for all developmental stages in all five transgenic lines.

**Quantitative real time PCR (qRT-PCR) of *kin-19*.** Synchronized populations of N2/wildtype were grown at 25°C on OP50, and harvested every 2 hours post-plating, starting at 4 hrs and

extending to 40 hrs. Total RNA was extracted from each population using Trizol, cDNA was synthesized from the RNA and used for qRT-PCR. *kin-19* and *ama-1* specific primers were used for each qRT-PCR run with SYBR Green on Biorad CFX96 RT-PCR detection system.

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